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NANCY JANE FLAUMAN

(Typed or Printed Name of Person Mailing Paper or Fee)

Nancy Jane Flauman
(Signature of Person Mailing Paper or Fee)

1900-0252.21
PATENT

RECOMBINANT TECHNIQUES FOR PRODUCTION
OF BRAIN NATRIURETIC PEPTIDE

Cross-Reference to Related Application

^{SUB A1} This is a continuation-in-part of U.S. Serial No. 206,470, filed 14 June 1988, which is a continuation-in-part of U.S. Serial No. 200,383, filed 31 May 1988.

Technical Field

The invention relates generally to natriuretic and homologous peptides found in brain and cardiac tissue. More particularly, it relates to the gene encoding a natriuretic peptide obtained from porcine brain and genes encoding peptides related by amino acid sequence in other species.

Background Art

The existence of peptides in the atrium which are responsible for maintenance of normal extracellular fluid parameters--i.e., the volume and pressure of liquid in the blood vessels--is well known. A series of closely related peptides, designated atrial natriuretic peptides, have been isolated from several species and identified, and analogs of these peptides have been prepared.

The natriuretic effect of a crude extract of rat atrial tissue was demonstrated over seven years ago. A number of peptides with diuretic and natriuretic properties have since been isolated from atrial tissue and sequenced: Flynn, T.G., et al., Biochem Biophys Res

Commun (1983) 117:859-865; Currie, M.G., et al., Science (1984) 223:67-69; Kangawa, K., et al., Biochem Biophys Res Commun (1984) 118:131-139; U.S. patent 4,496,544; U.S. patent 4,508,712; Kangawa, K., et al., Biochem Biophys Res Commun (1984) 119:933-940; Garcia, R., et al., Biochem Biophys Res Commun (1985) 126:178-174; Katsube, N., et al., Biochem Biophys Res Commun (1985) 128:325-330; U.S. patents 4,607,023; 4,557,864; and 4,618,600; copending applications 616,488; 766,030; and 870,795. These peptides, called atrial natriuretic peptides (ANPs), are cyclic disulfides comprising 17 amino acids in the cycle (including the two cysteines which provide the disulfide bond). The gene which encodes them encodes a much longer protein which is then processed into shorter versions which make up the set of ANPs.

Various analogs of the isolated atrial peptides are also described in copending applications 921,360; 138,893; and 174,739.

It is understood that these peptides and their analogs are effective in regulating blood pressure by controlling fluid volume and vessel diameter. A number of disease states are characterized by abnormal fluid retention, including congestive heart failure, cirrhosis of the liver, and nephrotic syndrome. These diseases are associated with excessive fluid accumulation on the venous side of circulation, and an underperfusion of the kidneys, leading to a fall in glomerular filtration rate (GFR). In addition, reduced renal perfusion stimulates secretion of renin, a proteolytic enzyme whose which, in the circulation, leads to the formation of angiotensin, a powerful constrictor of the arteriole. Renin also stimulates release of the sodium-retaining hormone aldosterone by the adrenal gland.

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102
hANP Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-
pBNP Asp-Ser-Gly-Cys-Phe-Gly-Arg-Arg-Leu-Asp-Arg-Ile-
*1 * *

35

There are nine (starred) positions which are not homologous. The conservative substitution of Leu for Ile or Met, found in rat or human ANP sequences, respectively, is a known acceptable substitution.

SUB B¹
10 Subsequent papers from this same group at Miyazaki Medical College further characterize these proteins. Sudoh, T., et al., Biochem Biophys Res Comm (1988) 155:726-732, report the isolation of a 32-amino acid natriuretic peptide ("BNP-32") from porcine brain which contains the 26 amino acids of the porcine BNP described above at its C-terminus and an additional N-terminal 6-amino acid extended portion of the sequence Ser-Pro-Lys-Thr-Met-Arg-. In papers following on
15 subsequent pages, levels of various natriuretic peptides in tissues are reported. Ueda, S., et al., (ibid.), pp. 733-739, utilized a radioimmunoassay to localize and measure the levels of porcine BNP and porcine BNP-32 in the brain and spinal cord. The results showed that both
20 BNP and BNP-32 were major forms of immunoreactive BNP in the porcine brain, and that the highest concentrations were found in the medulla-pons, striatum, and spinal cord. The porcine form of atrial natriuretic peptide (pANP) was also found in the porcine brain but at a level ap-
25 proximately 13 times lower than that characteristic of BNP. Minamino, N., et al. (ibid.), pp. 740-746, report the results of radioimmunoassay for porcine BNP and ANP in peripheral tissue. The concentration of BNP was highest in cardiac atrium of the tissues assayed. The
30 immunoreactive form of this protein was characterized as mostly a 12 kd high molecular weight form; less than 15% of the total immunoreactive BNP in atrial tissue is of the lower molecular weight forms pBNP or pBNP-32.

In a subsequent issue of this publication,
35 Minamino, N., et al., Biochem Biophys Res Comm (1988) 157:402-409, reported the isolation and characterization

of this higher molecular weight form of BNP from porcine heart. The complete amino acid sequence of this protein was obtained and shown to contain the 26-amino acid pBNP (and 32-amino acid pBNP-32) at its carboxy terminus. The full-length protein contains 106 amino acids. Finally, Maekawa, K., et al. (ibid.), pp. 410-416, report the cloning and sequence analysis of a cDNA encoding a precursor protein for porcine BNP. A cDNA library was obtained from porcine cardiac atrium and the relevant BNP-encoding gene was isolated and sequenced. The gene was found to include a 25-residue putative signal peptide at the N-terminus followed by the codons corresponding to the 106 amino acids of the reported protein.

These results are consistent with the information available from studies of the atrial-derived natriuretic peptides which are generally also associated with longer precursors. In the parent application herein, the gene encoding porcine BNP was provided, which permitted the putative amino acid sequence of the upstream portion of these precursor proteins to be deduced. While the cDNA obtained in the parent application was incompletely processed and contained an intron, further manipulation of this sequence using standard techniques as described below permitted the location of the intron to be established. Furthermore, the availability of the cDNA encoding pBNP permitted, with considerable effort and ingenuity as shown below, retrieval of genes encoding proteins of similar amino acid sequences from human and dog genomic libraries. Accordingly, the invention provides access to a family of natriuretic peptides (NPs) and natriuretic-related peptides (NRPs) from a variety of vertebrate sources.

Disclosure of the Invention

The invention provides the complete gene sequence for pBNP and the prepro form thereof and thus the

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sub
B9
15
dir
sequ

SUB
BIO 25



35

Gly-;

Ser-Gly-;

5 Asp/
Lys/ -Ser-Gly-;
Gly

Arg/
His/ - Lys/ -Ser-Gly-;
Gln Gly

10 Met/ - Arg/ Asp/
Val Gln Lys/ -Ser-Gly-;
Gly

Thr/ - Met/ Arg/ Asp/
Met Val Gln Lys/ -Ser-Gly-;
Gly

15 Lys- Thr/ - Met/ Arg/ Asp/
Met Val Gln Lys/ -Ser-Gly-;
Gly

Pro-Lys- Thr/ - Met/ Arg/ Asp/
Met Val Gln Lys/ -Ser-Gly-;
Gly

20 Ser-Pro-Lys- Thr/ - Met/ Arg/ Asp/
Met Val Gln Lys/ -Ser-Gly-;
Gly

or a 10- to 109-amino acid sequence shown as the native upstream sequence for porcine, canine or human BNP in Figure 8, or a composite thereof;

25 R^2 is (OH), NH_2 , or $NR'R''$ wherein R' and R'' are independently lower alkyl (1-4C) or are

Asn/
Lys

Asn/ -Val
Lys

Asn/ -Val-Leu
Lys

Asn/ -Val-Leu-Arg
Lys

35 Asn/ -Val-Leu-Arg- Arg/
Lys Lys

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B

Asn/ -Val-Leu-Arg- Arg/ - Tyr/
Lys Lys His

or the amides (NH₂ or NR'R") thereof,
with the proviso that if formula (1) is

R¹-Cys-Phe-Gly-Arg-Arg-Leu-Asp-Arg-
Ile-Gly-Ser-Leu-Ser-Gly-Leu-Gly-Cys-R²

and R¹ is Asp-Ser-Gly-, R² cannot be Asn-Val-Leu-Arg-Arg-
Tyr.

In other aspects, the invention is related to
recombinant DNA sequences encoding the foregoing peptides
and to recombinant expression systems capable of produc-
tion of these peptides in suitably transformed hosts. The
invention is also related to methods to produce the
peptides of the invention using recombinant means by
culturing the transformed cells and recovering the desired
peptide from the cell cultures.

The invention is also directed to modified forms
of this class of peptides wherein 1 or 2 of the positions
contain conservative amino acid substitutions.

The invention also relates to pharmaceutical
compositions and methods of treatment using the peptides
of the invention.

Brief Description of the Drawings

Figure 1 shows the complete sequence of a
retrieved cDNA in unprocessed form which encodes porcine
BNP. The portion of the sequence which encodes the 26-
amino acid pBNP peptide is underlined and consists of
residues 660-723 and 1276-1289 inclusive.

Figure 2 shows oligonucleotides synthesized as
probes for pBNP-encoding cDNA.

Figure 3 shows the cDNA of Figure 1 with the
location of the additional intron established.

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Figure 4 shows the coding portions of the pBNP-encoding cDNA absent the introns.

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SUB B16
Figure 5 shows the DNA and deduced protein sequence for the coding portions of the gene encoding a canine protein with natriuretic activity.

Figure 6 shows Southern blots of human genomic DNA probed with human ANP (left panel) and with canine NRP (right panel).

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SUB B17
Figure 7 shows the DNA and deduced amino acid sequence of the human genomic clone encoding the human NRP.

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SUB B18
Figure 8 shows a comparison of the amino acid sequences of the prepro forms of the porcine, canine and human proteins of the invention.

Modes of Carrying Out the Invention

A. Definitions

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SUB B19
As used herein, "brain natriuretic peptide (BNP)" refers to an amino acid sequence which is encoded by a DNA capable of hybridizing to an effective portion of the DNA shown in Figure 1 under defined stringency conditions, and which has natriuretic activity. It is believed that the brains of all vertebrates contain a subpopulation of peptides with this activity which comprise peptides analogous to that disclosed herein as pBNP and longer precursor proteins containing this amino acid sequence, as well as active fragments thereof.

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SUB B20
As used herein, "porcine brain natriuretic peptide (pBNP)" refers to the 26 amino acid sequence isolated by Sudoh et al., and set forth hereinabove. "pBNP-encoding cDNA" refers to the nucleotide sequence shown in Figure 1 herein, comprising residues 660-723 and 1276-1289 inclusive. The separation in the cDNA of the pBNP codons is presumably due to incomplete processing of

35

5 the mRNA which formed the template for this particular clone. This clone was deposited at the American Type Culture Collection, Rockville, MD, on 10 June 1988 and has accession number ATCC 40465.

10 The pBNP-encoding cDNA shown in Figure 1, because it contains additional sequences encoding precursor proteins, and, as explained below, presumably contains nucleotides corresponding to an additional intron besides that represented by the sequence separating the pBNP-encoding portion per se, can be used as an effective probe to obtain either genomic or cDNA sequences encoding corresponding associated brain natriuretic peptides in various vertebrate species. "Precursor brain natriuretic peptide" as used in the present application refers to peptides with natriuretic activity encoded by the gene sequence from which, for example, the pBNP protein is derived but processed so as to obtain peptides of different length. Similar processing differences presumably exist in other vertebrates as well; the entire class of such natriuretic peptides is retrieved by the DNA probe of the invention. For example, examination of the reading frame of the pBNP-encoding DNA shows an N-terminal extension so that N-terminally extended peptides can be postulated. It has been shown that the ANP precursor "pro-ANP" is processed differently in atrial and brain tissues leading to different ANP peptides. By analogy to the peptides found in the atrium, it is postulated that an important peripheral form of BNP would be the 29-residue peptide of pBNP N-terminally extended with the tripeptide Thr-Met-Arg. Further N-terminal extended peptides with the additional upstream residues Ser-Pro-Lys and Gly-Ile-Arg-Ser-Pro-Lys are also expected. Thus, examination of the reading frame which contains the pBNP also permits postulation of additional upstream processing sites which would extend the N-terminal sequence further.

5 Other extended precursor peptides are discover-
able through standard techniques using the sequence
information of Figure 1. It is clear, by analogy with
atrial natriuretic peptide precursors, that the start of
the longest precursor, perhaps including a signal
sequence, is at the methionine codon shown in the upper-
most reading frame in the line spanning nucleotide 61 and
120, or at the closely positioned downstream ATG.
10 Therefore, it is clear that the reading frame is not
maintained from this start of translation into the pBNP-
encoding region. This indicates that there is at least
one other intron transcribed into the cDNA clone
retrieved. The location of this intron and deduction of
15 the full sequence for the longest form of precursor
peptide is described in further detail below. In any
event, precursor BNP peptides associated with pBNP include
other natriuretic peptides encoded by this depicted gene;
analogous groups of peptides are collectively designated
20 natriuretic "NP" peptides in other species.

Additional terminology which is useful is the
term "prepro" NP, which refers to the encoded peptide hav-
ing both the native associated signal sequence which ef-
fects secretion of the various forms of the peptide with
natriuretic activity and an amino acid sequence of the
25 secreted peptide which is fused upstream of the cyclic
portion absolutely required for this activity. The "pro"
form having the upstream sequence may represent the
circulating form of the peptide. With respect to the
three specific embodiments included within the present
invention, which are shown in detail in Figures 3, 5, and
7 for porcine, canine and human proteins, respectively,
the location of the putative signal sequences representing
the "pre" sequence is shown in each figure, as well as the
35 full-length mature protein, which is thought to be a pre-
cursor form designated the "pro" form. Because various

processing sites are available, as indicated by the upward-pointing arrows in these figures and in the composite sequences shown in Figure 8, attempts to make a fine-line and definite distinction between the "pro" NP and "NP" are probably meaningless. The peptides defined by the invention are set forth in formula (1) above and have natriuretic activity, regardless of the length of the N-terminal form preceding the identified 26-amino acid regions corresponding to the porcine "BNP" of Sudoh, or attached to the cyclic portion thereof.

"Expression system" refers to a DNA which contains a coding region operably linked to suitable control sequences capable of effecting its expression in a compatible host. Expression systems invariably comprise a promoter, but, depending on the host intended, may contain additional critical DNA such as ribosome binding site or CAP site, termination sequence, and optional enhancer sequences upstream from the promoter or in other operable locations. The recombinant expression systems of the invention herein comprise a DNA of the invention encoding a BNP, for example, a BNP derived from a vertebrate source, operably linked to additional DNA sequences which are capable of effecting its expression. The expression system may reside on a transfer vector such as a plasmid or a viral vector which is self-replicating independently of the chromosome of the host cell, or may be constructed so that when inserted into a host cell it is able to integrate into the chromosome.

B. Other Associated Porcine BNPs and Retrieval of Vertebrate NRP Genes

The invention, in one aspect, is directed to all members of the group of porcine BNP proteins encoded in the cDNA shown in Figure 1, and to conservative modifications thereof. The deduction of the amino acid sequence

Sub
B24

501B
B25
25

Sub
B²_u khm/19

that found in atrial natriuretic peptide precursors, as described by Greenberg et al., Nature (1984) 312:656-658, was easily obtained. As shown in Figure 4, which represents solely the portions shown as coding sequences of Figure 3, a reading frame of 131 amino acids is obtained. It is believed that the signal sequence is represented by amino acids 1-25, and that the cleavage site converting the prepro form of porcine BNP to the pro form is between Ser₂₅ and His at position 26 of the prepro sequence, as shown in Figure 4. The sequence of the porcine BNP reported by Sudoh having 26 amino acids is represented by amino acid residues 81-106 (106-131):

In addition to providing access to the class of porcine BNPs encoded on the retrieved cDNA, the cDNA of Figure 1 provides access to the corresponding precursor gene encoding the class of associated NRP proteins from various vertebrate species.

The pBNP-encoding cDNA shown in Figure 1, or an effective portion thereof can be used as a probe in gene libraries obtained from other vertebrate hosts, by a number of procedures generally known in the art. The source of the desired genes can be either a genomic library appropriate to the vertebrate species or a cDNA library from cells synthesizing the peptide. As explained below, although these peptides are synthesized in brain, they are also known to occur in atrial tissue at a lower level than the normally produced ANP. Therefore, the more readily accessible atrial tissue can also be used to prepare the cDNA library to be probed in preference to brain tissue. In this instance, high stringency and +/- hybridization can be used to distinguish the more pre-dominant ANP-encoding DNA. Preparation of both genomic and cDNA libraries is well known in art; indeed, some genomic libraries are commercially available. Preferred techniques for preparing cDNA libraries are disclosed by

Hyunh, V.T., et al., DNA Cloning Techniques--A Practical Approach (IRL Press, Oxford, 1984), and by Okayama and Berg, Mol Cell Biol (1983) 3:280-289. Preferably, the
5 procedure exemplified below can, for example, be followed.

The genomic or cDNA library is then probed under nonstringent conditions (e.g., 20% formamide, 6 x SSC at 37°C) to obtain hybridizing sequences. The retrieved
10 sequences can then be analyzed and sequenced according to standard procedures.

The entire pBNP-encoding DNA of Figure 1 can be used as a probe, or an effective portion can be used. What constitutes an effective portion depends on the
15 nature of the library being probed and can be determined experimentally. In general, if a genomic library is the source for retrieval of the desired gene, a segment of the pBNP-encoding cDNA extending from about residue 601-1300 is convenient. This segment bridges the intron which
20 interrupts the coding sequence for the pBNP protein. Upstream portions of the sequence could also be used. Of course, there is no particular disadvantage in using the entire clone. On the other hand, if cDNA libraries are being investigated, it may be desirable to use only a por-
25 tion of the cDNA which represents the ordinarily spliced coding regions. Thus, for example, a convenient probe might be a contiguous DNA sequence representing the pBNP codons (positions 660-723 and 1276-1289) or a somewhat smaller segment thereof.

It is understood, of course, that the actual
30 probes may be sequences shown, or preferably their complements.

Sub
P28
I35
In practice, the porcine DNA of Figure 1 was able to retrieve genes encoding related proteins in genomic libraries from a variety of other species, either directly or indirectly. Genomic libraries from pig, rat,

dog, cat and rabbit showed the ability to hybridize to the probe of Figure 1 under at least one of the conditions:

- (1) 50% formamide, 6 x SSC, 5 x Denhardt's, 10 mM sodium phosphate, 10 ug/ml sheared DNA at 42°C; and
- (2) 20% formamide, 6 x SSC, 5 x Denhardt's, 10 mM sodium phosphate, 10 ug/ml sheared DNA at 37°C.

Under both hybridization conditions, washing was at 1 x SSC, 0.1% SDS at 50-60°C for 1 hour.

Human genomic DNA did not hybridize to the DNA sequence of Figure 1 under these conditions, but could be obtained indirectly using the other mammalian DNAs obtained using this probe.

By obtaining the canine DNA through use of the porcine probe, an insert having the sequence shown in Figure 5 was obtained, was designated pdBNP-1, and was deposited at the American Type Culture Collection on 14 December 1988 under Accession No. ATCC-67862. Using this sequence as a probe, a clone obtained from EcoRI-digested human genomic DNA was found which encodes a similar protein having natriuretic activity. This human DNA has the sequence shown in Figure 7, was designated phBNP-1, and was deposited at the American Type Culture Collection on 14 December 1988 under Accession No. ATCC-67863.

The amino acid sequence encoding the putative prepro forms of peptides with natriuretic activity from porcine, canine, and human species are shown in Figure 8. It is apparent that the porcine and canine species are more homologous in the region putatively responsible for natriuretic activity than the human sequence. Using the information in Figure 8, a class of peptides having natriuretic activity can be defined. This class is of the formula:

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SUB
B20
cont'd

SUB
B29

SUB
B30

SUB
B31

SUB
B32⁵

(1)

(H) ;

Gly-;

Ser-Gly-;

Asp/
Lys/ -Ser-Gly-;
Gly

Arg/ Asp/
His/ - Lys/ -Ser-Gly-;
Gln Gly

Met/ - Arg/ Asp/
Val Gln Lys/ -Ser-Gly-;
Gly

Thr/ - Met/ - Arg/ Asp/
Met Val Gln Lys/ -Ser-Gly-;
Gly

Lys- Thr/ - Met/ - Arg/ Asp/
Met Val Gln Gly -Ser-Gly-;

Pro-Lys- Thr/ - Met/ - Arg/ Asp/
Met Val Gln Gly -Ser-Gly-;

Ser-Pro-Lys- Thr/ - Met/ - Arg/ Asp/
Met Val Gln Gly -Ser-Gly-;

35

R^2 is (OH), NH_2 , or $NR'R''$ wherein R' and R'' are independently lower alkyl (1-4C) or is

5

Asn/
Lys

Asn/ -Val
Lys

Asn/ -Val-Leu
Lys

Asn/ -Val-Leu-Arg
Lys

Asn/ -Val-Leu-Arg- Arg/
Lys Lys

15

Asn/ -Val-Leu-Arg- Arg/ - Tyr/
Lys Lys His

or the amides (NH_2 or $NR'R''$) thereof,
with the proviso that if formula (1) is

20

R^1 -Cys-Phe-Gly-Arg-Arg-Leu-Asp-Arg-
Ile-Gly-Ser-Leu-Ser-Gly-Leu-Gly-Cys- R^2

and R^1 is Asp-Ser-Gly-, R^2 cannot be Asn-Val-Leu-Arg-Arg-Tyr.

25

As used above, "composite" of the sequences shown as native upstream sequences for porcine, canine or human BNP in Figure 8 refers to upstream sequences as there shown, where each position contains the alternative amino acids shown for all three species interchangeably.

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The composites of these sequences are constructed as were the composites for the specifically designated sequences in formula (1) and definitions of R^1 and R^2 above.

In addition, these peptide sequences can be modified by substituting one or two conservative amino acid substitutions for the positions specified, including
35 substitutions which utilize the D rather than L form. As

these peptides can be synthesized using standard solid-phase techniques, for example, it is not necessary to confine the conservative substitutions to amino acids encoded by genes.

Upon retrieval of the precursor gene encoding the BNP family for a particular vertebrate species, deduction of the BNP peptides associated with that species is a matter of translation of the determined sequence and identification of the processing site. Guidance is given by virtue of the pattern with respect to the atrial natriuretic peptide counterparts. It is believed that in analogy to the atrial peptides, the BNP proteins are cyclic disulfides formed by oxidation of the cysteine residues at positions 4 and 20 of the sequence shown above for pBNP. The class of BNP peptides encoded for a particular species is believed to include truncated forms exocyclic to this disulfide bonded ring as well as extended forms of the pBNP shown, including peptides with one or two conservative amino acids substitutions in the sequence.

A deduced (or otherwise generated) peptide sequence falls within the scope of certain natriuretic proteins of the invention, provided that the DNA encoding it directly or indirectly hybridizes to the pBNP-encoding cDNA of Figure 1 under conditions corresponding to the stringency represented by hybridization in buffer containing 20% formamide, 5 x Denhardt's, 6 x SSC, 100 mg/ml RNA, and 0.05% sodium pyrophosphate at 42°C, followed by washing at 60°C at 1 x SSC, 0.1% SDS, or under conditions (1) or (2) described above. In addition, the peptide encoded by this DNA must exhibit natriuretic activity assayed as described below.

By "direct hybridization" is meant that the DNA encoding the natriuretic peptide hybridizes to the DNA shown in Figure 1 or an effective portion thereof per se.

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B36

SUB
B37

SUB
B 31

By "indirect hybridization" is meant that the DNA hybridizes to an DNA which is capable itself of hybridizing to the porcine BNP of Figure 1. Thus, the human sequence shown in Figure 7 indirectly hybridizes to the porcine BNP through the canine sequence of Figure 5.

10 The invention is also directed to modified forms of the BNP proteins encoded by the cDNA of Figure 1. One or two of the positions of these BNPs can be altered, so long as activity is retained. Conservative amino acid substitutions are preferred--that is, for example, aspartic/glutamic as acidic amino acids; lysine/arginine/histidine as basic amino acids; leucine/isoleucine, methionine/valine as hydrophobic amino acids; serine/glycine/alanine/threonine as hydrophilic amino acids. However, as the peptides need not be prepared by re-combinant methods or from the gene, the substitutions may include nonencoded amino acids such as the D- or beta-amino forms.

15

20 B. Production of BNP

The BNP protein of the invention can be produced in a variety of ways, including using recombinant methods.

25 The retrieved genes encoding BNP peptides can then be manipulated and expressed using a variety of recombinant systems. Peptides having the sequence encoded by any subsegment of the retrieved gene can be obtained in host systems which do not process the translated protein by proper design of the expression system. For example, 30 the expression system is constructed by placing an ATG start codon immediately preceding the desired N-terminus and a termination codon after the desired C-terminus, with appropriate modification of any adjacent sequences. The desired coding sequence is then ligated in operable linkage to a control system functional in procaryotic or 35

eucaryotic hosts, as desired. A large number of control systems are now known in the art.

As the natriuretic peptide precursors are
5 evidently processed in certain eucaryotic systems, attention should be paid to the choice of the recombinant host, or it is possible to prevent processing by modification of the gene sequence so as to encode substitute amino acids in positions believed to be susceptible to cleavage by
10 proteolytic enzymes. For example, the arginine immediately upstream from the aspartic acid residue at position one of pBNP could be replaced by a threonine residue, thus rendering the resulting peptide non-susceptible to trypsin cleavage at that site. In the alternative,
15 expression can be effected in hosts which are deficient in enzymes capable of processing these peptides.

As the genes encoding the families of natriuretic-related peptides for various vertebrate species are made accessible by the availability of the probes
20 constructed from pBNP-encoding DNA, these genes can be manipulated by replacing the codons for one or more amino acids by site directed mutagenesis, to obtain sequences encoding analogs of these peptides which retain natriuretic activity.

25 Construction of expression vectors and recombinant production from the appropriate DNA sequences are performed by methods known in the art per se.

Expression can be in procaryotic or eucaryotic systems. Procaryotes most frequently are represented by
30 various strains of E. coli. However, other microbial strains may also be used, such as bacilli, for example Bacillus subtilis, various species of Pseudomonas, or other bacterial strains. In such procaryotic systems, plasmid vectors which contain replication sites and
35 control sequences derived from a species compatible with the host are used. For example, E. coli is typically

transformed using derivatives of pBR322, a plasmid derived from an E. coli species by Bolivar et al., Gene (1977) 2:95. Commonly used procaryotic control sequences, which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, including such commonly used promoters as the beta-lactamase (penicillinase) and lactose (lac) promoter systems (Chang et al., Nature (1977) 198:1056) and the tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res (1980) 8:4057) and the lambda-derived P_L promoter and N-gene ribosome binding site (Shimatake et al., Nature (1981) 292:128). Any available promoter system compatible with procaryotes can be used.

The expression systems useful in the eucaryotic hosts comprise promoters derived from appropriate eucaryotic genes. A class of promoters useful in yeast, for example, include promoters for synthesis of glycolytic enzymes, including those for 3-phosphoglycerate kinase (Hitzeman et al., J Biol Chem (1980) 255:2073). Other promoters include those from the enolase gene (Holland, M.J., et al. J Biol Chem (1981) 256:1385) or the Leu2 gene obtained from YEp13 (Broach, J., et al., Gene (1978) 8:121).

Suitable mammalian promoters include metallothionein, the early and late promoters from SV40 (Fiers et al., Nature (1978) 273:113) or other viral promoters such as those derived from polyoma, adenovirus II, bovine papilloma virus or avian sarcoma viruses. Suitable viral and mammalian enhancers are cited above. In the event plant cells are used as an expression system, the nopaline synthesis promoter is appropriate (Depicker, A., et al., J Mol Appl Gen (1982) 1:561).

The expression system is constructed from the foregoing control elements operably linked to the BNP

sequences using standard methods, employing standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or
5 synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the
10 art, and the particulars of which are specified by the manufacturer or these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 ug of plasmid or DNA sequence is
15 cleaved by one unit of enzyme in about 20 ul of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about 1 hr to 2 hr at about 37°C are workable, although variations can be
20 tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol followed by running over a Sephadex G-50 spin column. If desired, size separation of the cleaved fragments may be performed
25 by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separation is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended
30 by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min. at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM MgCl₂, 6 mM DTT and 5-10 uM dNTPs.
35 The Klenow fragment fills in a 5' sticky ends but chews back protruding 3' single strands, even though the four

dNTPs, are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated followed by running over a Sephadex G-50 spin column. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion.

Synthetic oligonucleotides are prepared using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 0.1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles ³²P-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Ligations are performed in 15-30 ul volumes under the following standard conditions and temperatures: 20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM DTT, 33 ug/ml BSA, 10 mM-50 mM NaCl, and either 40 uM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 ug/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10- to 30-fold molar excess of linkers) are performed at 1 uM total ends concentration.

In vector construction employing "vector fragments," the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and prevent religation of the vector. BAP digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na⁺ and Mg⁺² using about 1 unit of BAP per ug of vector at 60°C for about 1 hr. In

order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated and desalted by application to a Sephadex G-50 spin column. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

For portions of vectors derived from cDNA or genomic DNA which require sequence modifications, site specific primer directed mutagenesis is used. This is conducted using a primer synthetic oligonucleotide complementary to a single stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a stand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The resulting plaques are hybridized with kinased synthetic primer at a temperature which permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques which hybridize with the probe are then picked, cultured, and the DNA recovered. Details of site specific mutation procedures are described below in specific examples.

Correct ligations for plasmid construction can be confirmed by first transforming E. coli strain MM294 obtained from E. coli Genetic Stock Center, CGSC #6135, or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or

other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmid from the transformants are then
5 prepared according to the method of Clewell, D.B., et al., Proc Natl Acad Sci USA (1969) 62:1159, optionally following chloramphenicol amplification (Clewell, D.B., J Bacteriol (1972) 110:667). The isolated DNA is analyzed
10 by restriction and/or sequenced by the dideoxy method of Sanger, F., et al., Proc Natl Acad Sci USA (1977) 74:5463 as further described by Messing et al., Nucleic Acids Res (1981) 9:309, or by the method of Maxam et al., Methods in Enzymology (1980) 65:499.

15 The constructed vector is then transformed into a suitable host.

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N., Proc Natl Acad Sci USA (1972) 69:2110, or the RbCl method described in Maniatis et al., Molecular Cloning: A Laboratory Manual (1982), Cold Spring Harbor Press, p. 254, is used for procaryotes or other cells which contain substantial cell wall barriers. Infection with Agrobacterium tumefaciens (Shaw, C.H., et al., Gene (1983) 23:315) is used for certain plant cells.
25 For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 52:546, is preferred. Transformations into yeast are carried out according to the method of Van Solingen, P., et al., J Bacter (1977) 130:946, and Hsiao, C.L., et al., Proc Natl Acad Sci USA (1979) 76:3829.

30 The transformed cells are then cultured under conditions favoring expression of the BNP sequence and the recombinantly produced protein recovered from the culture.

35 In addition to recombinant production, peptides whose deduced sequences are sufficiently short to make

direct peptide synthesis practical can be prepared using standard solid-phase techniques.

Thus, compounds within the scope of the present invention can be synthesized chemically by means well known in the art such as, e.g., solid-phase peptide synthesis. The synthesis is commenced from the carboxy-terminal end of the peptide using an alpha-amino protected amino acid. t-Butyloxycarbonyl (Boc) protective groups can be used for all amino groups even though other protective groups are suitable. For example, Boc-Val-OH, Boc-Leu-OH, Boc-Arg-OH or Boc-Tyr-OH (i.e., selected BNP analog carboxy-terminal amino acids) can be esterified to chloromethylated polystyrene resin supports. The polystyrene resin support is preferably a copolymer of styrene with about 0.5 to 2% divinyl benzene as a cross-linking agent which causes the polystyrene polymer to be completely insoluble in certain organic solvents. See Stewart, et al., Solid-Phase Peptide Synthesis (1969), W.H. Freeman Co., San Francisco, and Merrifield, J Am Chem Soc (1963) 85:2149-2154. These and other methods of peptide synthesis are also exemplified by U.S. Patent Nos. 3,862,925; 3,842,067; 3,972,859; and 4,105,602.

The synthesis may use manual techniques or automatically employing, for example, an Applied Biosystems 430A Peptide Synthesizer (Foster City, California) or a Biosearch SAM II automatic peptide synthesizer (Biosearch, Inc., San Rafael, California), following the instructions provided in the instruction manual supplied by the manufacturer.

Of course, since automated synthesis also permits control of the sequence, the above-mentioned modifications to the amino acid sequence obtained by modifying the gene as described above are available using this method of synthesis. In addition, it is not necessary that the substituted amino acid be encoded by a gene.

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gene sequence capable of hybridizing directly or indirectly to pBNP-encoding DNA under the stringency conditions set forth herein, or be defined by formula (1) or its modified forms as set forth above, and must show activity in an in vitro receptor binding assay; either binding to the clearance receptors as shown by the competition assay, or to the effector receptors as shown by the alteration of cyclic GMP levels, or both. The peptide may or may not have direct biological activities associated with pBNP, such as cyclic GMP activity, if it inhibits clearance receptor binding in a manner that correlates with an in vivo test for natriuretic and diuretic activities. The peptides may also be vasodilators.

Receptor Binding Assays

Specific ANP receptor sites have been identified on target tissues, such as kidney, adrenal, blood vessels, and cultured cells. Napier, M.A., et al., Proc Nat Acad Sci USA (1984) 81:5946-5940; DeLean, A., et al., Endocrinology (1984) 115:1636-1638; Schenk, D.B., et al., Biochem Biophys Res Comm (1985) 127:433-442. Such tissues will have receptors for BNP binding which may or may not be identical to those for ANP. Since the binding of ANP or ANP analogs to these specific receptor sites is presumptively a prerequisite of biological activity, binding of BNP-associated peptides, or their modified forms to these receptors is considered predictive of biological activity.

An assay has been developed, generally in accordance with the disclosure of Schenk, supra, and Scarborough, R.M., et al., J Biol Chem (1986) 261:12960-12964, which evaluates the ability of ANP analogs to compete with a labeled native ANP for binding to cultured BSM and BAE cells. A similar assay, utilizing labeled pBNP can be used to evaluate candidate BNP family

peptides. The pBNP (shown above) was iodinated on the carboxy-terminal Y residue and is identified as (^{125}I)-pBNP. Analogous "competitive displacement" receptor binding assays are considered commonplace in the art for examining specific ligand-receptor interactions.

In this assay, 0.5 nM (^{125}I)-pBNP or (^{125}I)-human NRP is incubated in each individual sample of BASM cells in the presence of varying amounts of unlabeled pBNP or a candidate peptide encoded by a gene hybridizing to pBNP-encoding cDNA.

Increasing concentrations of pBNP, or successful candidate peptide effectively prevent (^{125}I)-pBNP binding to BASM cell-associated receptors. The concentration of unlabeled peptide at which 50% of maximal (^{125}I)-pBNP binding is displaced is called $K_i(\text{app})$ and reflects receptor-binding affinity. Therefore, a peptide with a $K_i(\text{app})=100\text{nM}$ displays substantially weaker interaction with a receptor than peptide with a $K_i(\text{app})=10\text{nM}$. Assuming these BNP analogs act at one or more BNP receptor sites, then increased receptor affinity should reflect increased biological potency.

The choice of the proper natriuretic peptide for the competition assay above should be made with regard to the peptide of the invention being tested. The receptors utilized by, for example, ANP and BNP may be the same or different. Alternate species forms of either may be used as competitors in suitable assay reactions for candidate peptides of formula (1).

Whole Mammal Bioassays

The biological activity of NP sequences of the present invention (which show activity in the receptor assay above), can be confirmed in anesthetized rats and dogs. The correlation of receptor binding affinity and in

vivo effects demonstrates the predictive value of the receptor assays for biological activity.

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1. Diuresis and Natriuresis in Anesthetized Rats

In one method, cannulae are placed in the left and right ureters and femoral vein of anesthetized rats and urine is collected from the ureters. NP compositions are administered via the femoral vein. Prior to infusing the NP, saline is infused for 30 minutes, urine is collected for 6 five-minute baseline periods and urine volume is determined gravimetrically.

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Following these baseline collection periods, various NPs are infused for 30 or 60 minutes and urine volume is measured in five-minute periods during infusion and for 60 minutes following infusion (at which time rats are returned to saline). Data are examined by averaging urine flow rates for six five-minute baseline control periods immediately preceding infusion, and comparing values during and after administration of NP with the "baseline" control values. Responses to NP are thus evaluated and plotted as the percent of baseline control responses. Responses to peptides that are substantially above baseline \pm SD can thus be interpreted as being statistically significant increases.

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2. Diuresis and Natriuresis in Anesthetized Dogs

The biological activity of NP of the present invention can also be confirmed in pentobarbital-anesthetized dogs. In these examples, cannulae are placed in the left and right ureters or urinary bladder and femoral vein of anesthetized dogs and urine is collected. NP is administered via the femoral vein. Prior to infusing NP, saline is infused for 30 minutes and urine is then collected for three ten-minute collection periods. Urine

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volume is determined gravimetrically and urine sodium is determined photometrically.

5 Following these three baseline collection periods, the selected NP is infused for 60 minutes and urinary flow and urinary sodium excretion measured for an additional 60 minutes following infusion. During infusion (60 minutes) and recovery (60 minutes), ten-minute collection periods are obtained. Control animals which received
10 only saline are studied in parallel.

Data are examined by comparing urine flow rates and sodium excretion rates for dogs infused with various NP portions against control animals infused with saline.

15 Isolated Tissue Bioassays

The effect of NP in vivo may be achieved solely by the ability to potentiate the effect of endogenous NP, through blockage of the receptors involved in binding and clearing endogenous NPs. To the extent that this is the
20 case for a particular NP, it could be expected that the diuretic and natriuretic effects of the NP would be diminished or eliminated in isolated tissue where NPs are not present unless specifically supplied.

25 Thus, NP compositions fall within the scope of the invention even if their activity in isolated tissue bioassays is low; however, activity in these assays may also be present.

30 D. Utility and Administration

Briefly, the natriuretic peptides of the invention are useful in treatment of disorders associated with high levels of extracellular fluids such as hypertension. The compounds are administered in conventional formulations for peptides such as those described in Remington's
35 Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa. (latest edition). Preferably, the peptides are

administered by injection, preferably intravenously, using appropriate formulations for this route of administration. Dosage levels are on the order of 0.01-100 ug/kg of subject.

These compounds, and compositions containing them, can find use as therapeutic agents in the treatment of various edematous states such as, for example, congestive heart failure, nephrotic syndrome and hepatic cirrhosis, in addition to hypertension and renal failure due to ineffective renal perfusion or reduced glomerular filtration rate. The natriuretic peptides of the invention are particularly effective in the treatment of congestive heart failure.

Thus the present invention also provides compositions containing an effective amount of compounds of the present invention, including the nontoxic addition salts, amides and esters thereof, which may, alone, serve to provide the above-recited therapeutic benefits. Such compositions can also be provided together with physiologically tolerable liquid, gel or solid diluents, adjuvants and excipients.

These compounds and compositions can be administered to mammals for veterinary use, such as with domestic animals, and clinical use in humans in a manner similar to other therapeutic agents. In general, the dosage required for therapeutic efficacy will range from about 0.001 to 100 ug/kg, more usually 0.01 to 100 ug/kg of the host body weight. Alternatively, dosages within these ranges can be administered by constant infusion over an extended period of time, usually exceeding 24 hours, until the desired therapeutic benefits have been obtained.

Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The

preparation may also be emulsified. The active ingredient is often mixed with diluents or excipients which are physiologically tolerable and compatible with the active ingredient. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH-buffering agents, and the like.

The compositions are conventionally administered parenterally, by injection, for example, either subcutaneously or intravenously. Additional formulations which are suitable for other modes of administration include suppositories, intranasal aerosols, and, in some cases, oral formulations. For suppositories, traditional binders and excipients may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10% preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained-release formulations, or powders, and contain 10%-95% of active ingredient, preferably 25%-70%.

The peptide compounds may be formulated into the compositions as neutral or salt forms. Pharmaceutically acceptable nontoxic salts include the acid addition salts (formed with the free amino groups) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may be derived from

inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino
5 ethanol, histidine, procaine, and the like.

In addition to the compounds of the present invention which display natriuretic, diuretic or vasorelaxant activity, compounds of the present invention can also be employed as intermediates in the synthesis of
10 such useful compounds. Alternatively, by appropriate selection, compounds of the present invention whose activity levels are reduced or eliminated entirely can serve to modulate the activity of other diuretic, natriuretic or vasorelaxant compounds, including compounds outside the
15 scope of the present invention, by, for example, binding to clearance receptors, stimulating receptor turnover, or providing alternate substrates for degradative enzyme or receptor activity and thus inhibiting these enzymes or receptors. When employed in this manner, such compounds
20 can be delivered as admixtures with other active compounds or can be delivered separately, for example, in their own carriers.

Compounds of the present invention can also be used for preparing antisera for use in immunoassays employ-
25 ing labeled reagents, usually antibodies. Conveniently, the polypeptides can be conjugated to an antigenicity-conferring carrier, if necessary, by means of dialdehydes, carbodiimide or using commercially available linkers. These compounds and immunologic reagents may be labeled
30 with a variety of labels such as chromophores, fluorophores such as, e.g., fluorescein or rhodamine, radioisotopes such as ^{125}I , ^{35}S , ^{14}C , or ^3H , or magnetized particles, by means well known in the art.

These labeled compounds and reagents, or labeled
35 reagents capable of recognizing and specifically binding to them, can find use as, e.g., diagnostic reagents. Samples

derived from biological specimens can be assayed for the presence or amount of substances having a common antigenic determinant with compounds of the present invention. In addition, monoclonal antibodies can be prepared by methods known in the art, which antibodies can find therapeutic use, e.g., to neutralize overproduction of immunologically related compounds in vivo.

Suitable subjects include those animals having conditions of high water or sodium ion accumulation. Both veterinary and therapeutic uses in humans are appropriate.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Retrieval of Porcine BNP-Encoding DNA

Porcine heart tissue frozen in liquid nitrogen was obtained and separated roughly into atrial and ventricular portions. Frozen atrial tissue (5 g) was first pulverized in a mortar and pestle, then ground to a powder in a tissuemizer and liquefied in a large volume (25 ml) of 5 M guanidinium thiocyanate containing 50 mM Tris (pH 7.5), 5 mM EDTA, and 5% beta-mercaptoethanol. Sarcosyl was then added to 2% and the sample was incubated at 65°C for 2 min whereupon insoluble material was removed by centrifugation at 7000 x g for 10 min. Total RNA was isolated from this supernatant by adding 2.5 g of CsCl, layering over a 10 ml cushion of 5.8 M CsCl and centrifuging again at 25,000 rpm for 12 hr. The supernatant was subsequently aspirated and the pellet dissolved in buffer containing Tris (50 mM), EDTA (5 mM), and beta-mercaptoethanol (2%). The RNA solution was then phenol extracted once and precipitated with ethanol. Poly A⁺ RNA was isolated by oligo dT cellulose chromatography.

Double-stranded DNA complementary to the porcine atrial mRNA (5 ug) was synthesized by the RNase H method. The cDNA was then methylated by standard methods and EcoRI linkers ligated and digested. The entire cDNA was then ligated into previously prepared lambda phage arms and packaged according to standard methods. Plating of this packaging reaction gave a library with $\sim 1.75 \times 10^5$ randomly isolated phage from this library showed that almost all (>95%) had inserts in the 1-5 kb range.

Probes were designed to detect the pBNP cDNA. As shown in Figure 2, conservation with respect to human ANP cDNA was assumed in constructing oligo 3351. Thus, the human ANP-encoding sequence shown in the figure was modified only to the extent required to obtain a sequence encoding pBNP. The second oligo, 3352, was another 60-mer designed according to mammalian codon preference and preferring G and T over A and C. The additional 60-mer 3376 was synthesized to match the human ANP sequence so as to eliminate false positives.

Approximately 300,000 phage from the above library were plated and lifted with nitrocellulose filters in duplicate. Series A (15 nitrocellulose filters) were denatured, neutralized, baked for 2 hr and prehybridized for 2 hr in hybridization buffer (20% formamide, 5 x Denhardt's solution, 6 x SSC, 0.05% pyrophosphate, 100 ug/ml salmon sperm DNA). Labeled oligonucleotide probe (3351, 1.5×10^7 cpm) was then added and the filters incubated overnight at 42°C. Filters were subsequently washed in 6 x SSC, 0.1% SDS at 20°C for 40 min., and then twice in 1 x SSC at 65°C for 10 min. Series B was treated in the same manner except oligo 3352 was used. Final washing was at 60°C in this case. In both cases filters were dried and subjected to autoradiography. Approximately 450 positives (0.2% of total clones plated) were obtained when probing with oligo 3351 and most of these are believed to be

porcine ANP based on previous screening with this oligo. Four clones in series B hybridized to oligo 3352. These hybrids were stable at 60°C but not at 65°C. Of these four, only clone 14 hybridized with oligo 3351 also, and this was picked and subjected to another round of purification. The purified phage was then grown, and the DNA was isolated by centrifugation at 36,000 rpm over a CsCl step gradient, phenol extracted, dialyzed, and ethanol precipitated. The phage DNA contained a 1.5 kb DNA insert when subjected to restriction analysis with EcoRI. This insert was then subcloned into an M13 sequencing vector and the sequence determined. The abundance of this BNP mRNA appeared about 400-fold lower than ANP in this library, or 0.0005%.

The DNA sequence of the insert from clone 14 is shown in Figure 1. The coding region for BNP is present within the clone; however, it is interrupted by what appears to be an intron at residue Val₂₂ of the 26-amino acid BNP. Therefore, it appears that this clone contains an unprocessed mRNA with one or more introns present.

Example 2

Identification of the Upstream Intron

As set forth above, the DNA sequence of Figure 1 shows a change in reading frame and, furthermore, by analogy to the ANP-encoding gene as described by Greenberg et al. (supra), may contain an upstream intron. In order to locate the position of this intron, the sequences surrounding its putative location were used as primers in an amplification procedure to obtain spliced DNA lacking the intron.

Poly A⁺ RNAs were isolated from porcine atrial tissue using the guanidinium isothiocyanate method of Chirgwin, J.M., Biochemistry (1979) 18:5294-5299, followed by oligo-dT cellulose chromatography. Approximately 2 ug

5 of the porcine atrial mRNA was incubated with 400 ng of
oligonucleotide 3895 (supra) as primer in a 20 ul reaction
containing 0.5 mM dNTPs, 50 mM Tris-HCl, pH 8.3, 10 mM
magnesium chloride, 10 units of RNasin, and 50 units of
reverse transcriptase. Subsequent amplification of the
resulting DNA was performed as described by Saiki, R.K.,
10 Science (1988) 239:487-491. After incubation for 1 hr at
37°C, half of the reaction was diluted to 100 ul in 67 mM
Tris-HCl, pH 8.8, 6.7 mM magnesium chloride, 16.6 mM am-
monium sulfate, 10 mM mercaptoethanol, 6.7 uM EDTA, 1 mM
dNTPs, 10% DMSO, and 400 ng of each primer oligonucleotide.
The oligonucleotide primers were those corresponding to
15 bases 100-123 (identity strand) and 652-685 (complementary
strand) of the pBNP clone shown in Figure 1.

The reaction mixture was denatured by boiling for
5 min followed by incubation at 42°C to allow primer an-
nealing. Thermus aquaticus polymerase (3 units) was added
and the sample further incubated at 72°C for 3 min. The
20 cycle (98°C, 1 min; 43°C, 1 min; 72°C, 3 min) was repeated
30 times without addition of additional polymerase. The
extended reaction was conducted under a 100 ul layer of
mineral oil, and a 10 ul aliquot was removed and analyzed
by standard agarose gel electrophoresis. The resulting DNA
25 fragments were visualized after staining with ethidium
bromide and purified by preparative agarose gel
electrophoresis. The amplified DNA fragment was then
kinased, ligated into M13, and sequenced.

Two DNA sequences resulted from this reaction:
30 one of approximate 650 bp was in low relative amount and
was presumably the unspliced version; the more abundant,
approximately 350 bp, band was assumed to be the fully
processed DNA and, indeed, showed the sequence set forth in
Figure 4 between the reverse arrows at positions 100-684.

Example 3

Retrieval of Additional BNP Encoding Genes

5 The entire 1504-base sequence in Figure 1, or a shorter segment constituting bases 601-1300 is then used as a probe to obtain the genes encoding the analogous BNP peptides and other vertebrate species. In this approach, blots of genomic DNA are used as substrate. Approximately 10 ug of genomic DNA from the liver of the appropriate species is digested with BamHI or PstI overnight, and the digested DNA precipitated with ethanol and electrophoresed on 0.8% agarose gels. The gels are blotted onto nitro-cellulose filters overnight, and the filters then de-natured, baked and prehybridized at 42°C in
10 prehybridization buffer (20% formamide, 5 x Denhardt's 6 x SSC, 100 mg/ml RNA, 0.05% sodium pyrophosphate).

The cDNA is labeled by nick-translation and hybridized to two panels from the same gel at 42°C overnight in the prehybridization buffer. The filters are
15 then washed in 1 x SSC, 0.1% SDS at 60°C and 65°C and exposed to autoradiographic film.

The genes encoding the analogous BNPs in the particular species are then amplified and sequenced according to standard techniques. The deduced sequences can be
20 manipulated to provide suitable restriction sites for insertion into expression systems and to provide desired stop and start codons by site-directed mutagenesis.

Example 4

Alternative Method to Obtain

Natriuretic Peptide-Encoding DNAs

30 In a modification of the method set forth in Example 3, both the canine and human genomic libraries yielded DNAs encoding natriuretic peptides.

35 Genomic DNA from pig, rat, dog, cat, rabbit and human organisms were probed on Southern blots using the

5 cDNA illustrated in Figure 1 herein under two different hybridization conditions:

(1) 50% formamide, 6 x SSC, 5 x Denhardt's, 10 mM sodium phosphate, 10 ug/ml sheared DNA at 42°C; and

(2) 20% formamide, 6 x SSC, 5 x Denhardt's, 10 mM sodium phosphate, 10 ug/ml sheared DNA at 37°C.

Washing in both cases was in 1 x SSC, 0.1% SDS at 50-60°C for 1 hr.

10 A dog genomic library obtained from Clontech Inc. yielded 2 clones under the condition (1) above and the DNA from these identified clones was digested with HaeIII or AluI and subcloned into M13. The resulting plaques were screened for hybridization to the porcine probe, and positive clones were sequenced. The identity of the clone was confirmed by detection of the BNP-encoding sequence of Figure 1, and the 2.9 kb HindIII fragment containing the entire gene was then subcloned into pBR322, and designated pdBNP-1. The DNA sequence of the portion of this clone encoding the BNP gene is shown in Figure 5, and the pdBNP-1 plasmid was deposited at the American Type Culture Collection on 14 December 1988 and has Accession No. ATCC-67862.

20 Although a human genomic library failed to yield signals corresponding to hybridization with the probe using the porcine DNA of Figure 1, use of pdBNP-1 as a probe under condition (1) above produced several distinct bands that could be visualized in blots of digested human genomic DNA, as shown in Figure 6. A preparative agarose gel was utilized to isolate EcoRI-digested human genomic DNA in the 6-7 kb size range, which isolated DNA was then cloned into lambda-ZAP2 (Stratagene Inc.), packaged, and the resulting mini-library was screened using the hybridization condition (1) above. Seven positive signals were purified and the insert subcloned into pBLUSCRIPT vector. The sequences of the M13 subclones of hybridization-positive HaeIII and AluI-digested plasmid DNA were determined. The sequence of

the coding region of the plasmid, phBNP-1, is shown in Figure 7 and the plasmid was deposited at the American Type Culture Collection on 14 December 1988 with Accession No. ATCC-67863.

Using the intron splice junction consensus sequences described by Mount, S., Nucleic Acids Res (1982) 10:459-472, it appears that the first exon of the human cDNA sequence contains two extra amino acids in the BNP precursor region as compared to the porcine sequence. This can be verified by PCR amplification of human atrial RNA.

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